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January 6, 2005

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Attn: TSCA Section 8(e)
U.S. Environmental Protection Agency
1201 Constitution Avenue, NW
Washington, DC 20460-0001

CONTAINS NO CBI

Subject: Notice in accordance with TSCA 8(e), *cis*-2-Pentenenitrile

Dear Sir or Madam:

This letter is to inform you of the results of a recently completed *in vitro* Mammalian Chromosome Aberration Test in Chinese Hamster Ovary Cells evaluating the above referenced substance. Under the conditions of this study, *cis*-2-Pentenenitrile was found to induce structural chromosome aberrations in Chinese Hamster Ovary Cells in the S9 activated test system, but not in the non-activated test system. *Cis*-2-Pentenenitrile was found to be negative for the induction of numerical chromosome aberrations in both the non-activated and S9 activated systems. The test results are considered to be positive in this *in vitro* test.

Questions regarding this submission should be addressed to the undersigned.

Sincerely,

Craig Mellinger

Craig Mellinger, C.I.H.
Manager, Occupational Health Compliance System

Enclosures

In Vitro Mammalian Chromosome Aberration Test in Chinese Hamster Ovary Cells
Unsanitized Version (31 pages)

282178

Study Title

H-26232:
In Vitro Mammalian Chromosome Aberration Test in Chinese Hamster Ovary Cells

TEST GUIDELINES: U.S. EPA Health Effects Test Guidelines, OPPTS 870.5375 (1998)

OECD Guidelines for the Testing of Chemicals, No. 473 (1998)

EC Commission Directive 2000/32/EC Annex 4D. No. L 136

AUTHOR: E. Maria Donner, Ph.D.

STUDY COMPLETED ON: December 15, 2004

PERFORMING LABORATORY: E.I. du Pont de Nemours and Company
Haskell[SM] Laboratory for Health and Environmental Sciences
Elkton Road, P.O. Box 50
Newark, Delaware 19714-0050

LABORATORY PROJECT ID: DuPont-15780

WORK REQUEST NUMBER: 15097

SERVICE CODE NUMBER: 531

SPONSOR: INVISTA
4123 East 37th Street North
Wichita, Kansas 67220

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in compliance with U.S. EPA TSCA (40 CFR part 792) Good Laboratory Practice Standards, which are compatible with the OECD Principles of Good Laboratory Practice (as revised 1997), ENV/MC/CHEM(98)17, OECD, Paris, 1998, except for the items documented below. The item listed did not impact the validity of the study.

1. The test substance was characterized by the sponsor prior to the initiation of this study. Although the characterization was not performed under Good Laboratory Practice Standards, the accuracy of the data is considered sufficient for the purposes of this study.

2. Neither the vehicle nor the positive controls were characterized by the testing facility or the sponsor. However, both the vehicle and positive controls were purchased from a reputable vendor and showed results consistent with historical control data.

3. The concentrations of the positive control and test substance dose solutions were not confirmed analytically; however, the solutions were prepared by trained personnel to ensure the accuracy of the concentrations.

Study Director: E. Maria Donner 15-Dec-2004

E. Maria Donner, Ph.D.
Senior Research Toxicologist and Manager
Haskell Laboratory for Health and
Environmental Sciences

Date

QUALITY ASSURANCE DOCUMENTATION

Work Request Number: 15097
Study Code Number: 531

The conduct of this study has been subjected to periodic Quality Assurance inspections. The dates of inspection are indicated below.

Phase Audited	***Audit Dates***	***Date Reported to Study Director***	***Date Reported to Management***
Conduct:	2 November 2004	2 November 2004	2 November 2004
Report/Records:	8-10 December 2004	10 December 2004	15 December 2004

Reported by: Annet L. Reigel 15-DEC-2004
Annet L. Reigel Date
Quality Assurance Auditor

CERTIFICATION

We, the undersigned, declare that this report provides an accurate evaluation of data obtained from this study.

Approved by: _____ 15-Dec-2004
Gary W. Jepson, Ph.D. Date
Research Manager

Issued by Study Director: _____ 15-Dec-2004
E. Maria Donner, Ph.D. Date
Senior Research Toxicologist and Manager

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STUDY INFORMATION

9th Collective Nomenclature: 2-Pentenenitrile, (2Z)-

Synonyms/Codes:
- cis-2-Pentenenitrile
- (Z)-2-Pentenenitrile
- 2-PN
- H-26232
- JO0501603 DRUM 1 (Lot No.)

Submitter's Notebook Number(s): E105688-25

Haskell Number: 26232

CAS Registry Number: 25899-50-7

Purity: 98.9%

Known Impurities: 2-methyl-3-butenenitrile (0.69%)
cis-2-methyl-2-butenenitrile (0.37%)
2,6-di-tert-butyl-p-cresol (inhibitor) 75 ppm

Physical Characteristics: Very pale yellow liquid

Stability: The test substance appeared to be stable under the conditions of the study; no evidence of instability was observed.

Study Initiated/Completed: October 1, 2004 / (see report cover page)

Experimental Start/Completion: October 27, 2004 / November 22, 2004

SUMMARY

The test substance, H-26232, was evaluated for its ability to induce chromosome aberrations in Chinese hamster ovary (CHO) cells *in vitro*. Numerical aberrations were recorded. The assessment was done both in the presence and absence of an exogenous S9 metabolic activation system. To establish a concentration range for the chromosome aberration assay, a preliminary toxicity assay was initially conducted.

The test substance was prepared in dimethyl sulfoxide (DMSO) as this vehicle was determined to be the solvent of choice based on solubility of the test substance and compatibility of the target cells. The test substance was soluble in the vehicle at approximately 500 mg/mL, the highest stock concentration prepared on the study. Dosing solutions were adjusted to compensate for the purity of the test substance.

In the preliminary toxicity and chromosome aberration assays, the cells were treated for 4 and 20 hours in the non-activated test system and for 4 hours in the S9 activated test system, and all cells were harvested at 20 hours after treatment initiation. The test substance was soluble in treatment medium at all dose levels tested. The final concentration of the vehicle (DMSO) in the treatment medium was 1%.

In the preliminary toxicity assay, the highest concentration tested was 810.5 µg/mL (10 mM), the guidelined limit dose for this test system (i.e., 5000 µg/mL or 10 mM, whichever is lower). The test substance was soluble in the vehicle and in the treatment medium at all concentration levels tested. The pH of the highest test substance concentration in medium was not significantly different than the vehicle control. No test substance precipitation was observed. Substantial toxicity (at least a 50% reduction in cell growth relative to the solvent control) was not observed at any concentration level in any test system. Based on the findings from the preliminary toxicity assay, the highest concentration initially chosen for the chromosome aberration assay was 810.5 µg/mL for all three test systems.

The concentrations chosen for the chromosome aberration assay were 324.2, 486.3, 648.4, 729.5 and 810.5 µg/mL for all three test systems. The osmolality of the highest test substance concentration in medium was not significantly different than the vehicle control. Substantial test substance-related toxicity (at least a 50% reduction in cell growth relative to the solvent control) or significant reduction in the mitotic index relative to the vehicle control was not observed at any concentration level in the 4- and 20-hour non-activated test systems. However, for the 4-hour activated test system, test substance-related inhibition of the mitotic activity was observed at all concentration levels. A repeat assay was conducted at a concentration range of 1.86 to 243.2 µg/mL. In this assay, a 49% reduction in cell growth relative to the solvent control was observed at 162.1 µg/mL.

Cytogenetic evaluations were conducted at 324.2, 648.4, and 810.5 µg/mL (10 mM) for the 4- and 20-hour non-activated test systems, and at 60.79, 121.6, 162.1 µg/mL (2 mM) for the 4-hour activated test system.

The percentage of cells with structural aberrations in the test substance-treated groups was not significantly increased above that of the solvent control at any concentration ($p > 0.05$, Fisher's exact test) in the 4- and 20-hour non-activated test systems. In the 4-hour S9 activated test system, the percentage of cells with structural aberrations in the 121.6 and 162.1 µg/mL test substance-treated groups was statistically significant ($p < 0.05$, Fisher's exact test). The Cochran-Armitage test for dose response was also statistically significant ($p < 0.05$). Although the observation of 4.5% of cells with structural aberrations at 121.6 µg/mL is within the historical solvent control range of 0-6% for structural aberrations, the 10.5% of cells with structural aberrations at 162.1 µg/mL, is above the historical solvent control range. Therefore, the data are considered biologically significant. The percentage of cells with numerical aberrations was statistically significant ($p < 0.05$, Cochran-Armitage test) in the non-activated test systems, but based on the low magnitude of the responses not considered biologically significant.

All criteria for a valid study were met. Under the conditions of this study, H-26232 was found to induce structural chromosome aberrations in the *in vitro* mammalian chromosome aberration test in Chinese hamster ovary cells in the S9 activated test system, but not in the non-activated test system. H-26232 was found to be negative for the induction of numerical chromosome aberrations in both the non-activated and S9 activated test systems. The test substance was concluded as positive in this *in vitro* test.

INTRODUCTION

The objectives of this study were to evaluate the ability of the test substance, H-26232, to induce structural chromosome aberrations in Chinese hamster ovary (CHO) cells *in vitro*. Numerical aberrations were recorded. The assessment was done both in the presence and absence of an exogenous S9 metabolic activation system.

MATERIALS AND METHODS

A. Testing Guidelines

This study was conducted in compliance with the following guidelines:

- U.S. Environmental Protection Agency (EPA), Health Effects Test Guidelines, OPPTS 870.5375, *In Vitro* Mammalian Chromosome Aberration Test. (1998).
- Ninth Addendum to the OECD (Organisation for Economic Cooperation and Development) Guidelines for the Testing of Chemicals, *In Vitro* Mammalian Chromosome Aberration Test, No. 473. (February, 1998)
- European Commission Directive 2000/32/EC Annex 4D. No. L 136.

B. Test Substance and Controls

1. Test Substance Identification: H-26232

The test substance was a very pale yellow liquid. The test substance batch used for this study was assigned the unique Haskell Identification Number 26232. The expiration data for the test substance is January 14, 2005. Additional information regarding the test substance is described on the Study Information page of this report.

2. Test Substance Characterization

The sponsor was responsible for determination and documentation of the analytical purity and composition of the test substance, and the stability and strength of the test substance in the solvent (or vehicle). Haskell Laboratory did not conduct analytical analyses of the dosing solution.

3. Sample Preparation, Stability, and Analytical Verification of Test Substance Concentrations

The sponsor-reported purity for H-26232 was 98.9% active ingredient. A correction factor of 1.011 was used for preparation of the dosing solutions. An analytical verification of the test substance concentrations was not conducted.

4. Controls

Negative:	Dimethyl Sulfoxide (DMSO, CAS# 67-68-5, ≥ 99% purity, Sigma)
Positive:	Mitomycin C (MMC, CAS# 50-07-7, Sigma)
	Cyclophosphamide (CP, CAS# 6055-19-2, Sigma)

The positive controls were dissolved in sterile water. The positive controls were assumed to be stable during this assay and no evidence of instability was observed.

C. Test System

The CHO-K_1 cell line was originally derived as a subclone from a parental CHO cell line. The cells require proline in the medium for growth, and have a modal chromosome number of 20. The population doubling time is 10-14 hours. The cell line was obtained from the American Type Culture Collection (ATCC number CCL 61), Manassas, Virginia. The karyotype and the absence of mycoplasm infection are routinely checked by Haskell Laboratory. This test system has been demonstrated to be sensitive to the clastogenic activity of a variety of chemicals.[1]

D. Experimental Design and Methodology

The study was conducted according to published procedures.[2-3] The test substance, as well as positive and negative (vehicle) controls, were administered in the presence and absence of an exogenous S9 metabolic activation system to cell cultures by addition to the culture medium. In the non-activated test system the treatment times were approximately 4 and 20 hours, and in the S9 activated test system approximately 4 hours. The dividing cells were arrested in metaphase approximately 18 hours after initiation of the treatment and harvested at approximately 20 hours. This harvest time point represents approximately 1.5 normal cell cycles, and is determined to ensure assessment of clastogenicity in first-division metaphase cells.[4] Cytogenetic analyses of structural chromosome aberrations were conducted on the 4- and 20-hour non-activated and the 4-hour activated assays. The cytogenetic assessment also included recording of numerical aberrations.

1. Solubility Determination and Selection of Solvent

Unless the sponsor specified the test substance vehicle or other appropriate solubility data was available, a solubility determination was conducted to determine the maximum soluble concentration of a workable suspension up to a maximum of 50 mg/mL for aqueous vehicles and 500 mg/mL for organic vehicles. Vehicles compatible with this test system, in order of preference, included, but were not limited to, culture medium or water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), or ethanol (CAS 64-17-5). The vehicle of choice for this study was DMSO, which permitted preparation of the highest workable/soluble stock concentration. Under the conditions of this test system, the final concentration of solvents other than water, physiological buffer, or medium did not exceed 1% of the treatment medium. After the addition of the dosing solution, the treatment medium was observed for precipitation (with the naked eye).

2. Exogenous Metabolic Activation

Liver homogenate (S9), prepared from male Sprague-Dawley rats induced with Aroclor 1254, were purchased commercially (Moltox, Inc., Boone, North Carolina) and stored frozen at approximately -70°C until used.

Immediately prior to use, the S9 was thawed and mixed with a cofactor pool to contain 100 µL S9/mL reaction mixture of approximately 4 mM NADP, 5 mM glucose-6-phosphate, 8 mM $MgCl_2$, 33 mM KCl, and 100 mM sodium phosphate buffer, pH 7.4. The S9 reaction mixture was stored on ice until used. The metabolic activity of the S9 was demonstrated by the response of the CP treated cultures.

3. Preparation of Target Cells for Toxicity and Chromosome Aberration Assays

Exponentially growing CHO-K_1 cells were seeded in labeled, sterile flasks. Approximately 5×10^5 cells/25 cm^2 flask were inoculated in complete medium (McCoy's 5A medium with 10% fetal bovine serum, 2 mM L-glutamine, 100 units penicillin/mL and 100 µg streptomycin/mL). Cultures were incubated at 37 ± 2°C in a humidified atmosphere of 5 ± 2% CO_2 in air for 16-24 hours.

4. Preliminary Toxicity Assay to Select Dose Levels

The selection of dose levels for the cytogenetics assay was based on a preliminary toxicity assay. At initiation of the assay, cell cultures seeded 16-24 hours earlier (at least one culture per concentration level) were exposed to at least 9 concentrations of the test substance and the vehicle control substance. The dividing cells were harvested at a single time point, approximately 20 hours from the initiation of treatment (~1.5 times the normal cell cycle). The exposure times were approximately 4 and 20 hours in the absence of S9 metabolic activation, and approximately 4 hours in the presence of S9 metabolic activation. After the 4-hour exposure period only, the treatment medium was removed and replaced with complete McCoy's 5A culture medium (Gibco). Approximately 20 hours after the initiation of exposure to the test substance, the cell cultures were microscopically inspected for the extent of monolayer confluency relative to the solvent control. Twenty hours after treatment initiation, the cells were harvested by trypsinization and counted by an automatic cell counter or by hemocytometer. Cell viability was not specifically assessed. The cell counts were used to determine cell growth inhibition relative to the solvent control.

Whenever possible, the highest concentration selected for the chromosome aberration assay induced at least a 50% cell growth inhibition relative to the vehicle control. At least two additional dose levels, demonstrating limited toxicity or no toxicity were also evaluated. In cases where there was little or no cytotoxicity, the highest dose level tested and at least two lower dose levels were selected for analysis. In cases where there was little or no cytotoxicity, but a precipitate was observed (with the naked eye) the lowest dose level demonstrating a precipitate and two other lower dose levels were selected for analysis.

If neither cytotoxicity nor precipitation was observed in the preliminary toxicity assay, the highest concentration did not exceed the limit dose of 5000 µg/mL or 10 mM, whichever was the

lower. The osmolality and pH of the vehicle control, as well as the highest soluble test substance concentration in the culture media, was determined. The osmolality was determined in connection with the main chromosome aberration assay, not in the preliminary toxicity assay. If the osmolality of the test concentrations exceeded the vehicle osmolality by 20% or more, the study director made a decision whether to take this into account when selecting the highest dose for the cytogenetics assay. If necessary, the pH of the treatment medium was adjusted to maintain a neutral pH based on a visual inspection. Precipitation was evaluated both at the beginning and the end of the treatment period by visual determination.

5. Frequency and Route of Administration for the Chromosome Aberration Assay

Cell cultures were treated once for approximately 4 hours in the absence and presence of S9 metabolic activation, and for 20 hours in the absence of metabolic activation. The test substance was added to the treatment medium in a test system compatible vehicle. This frequency and route of administration has been demonstrated to be effective in the detection of chemically induced mutagenesis in this test system.[2-3]

Based on OECD 473, a clear positive response did not require verification. Negative results did not require confirmation, but were justified.

6. Controls

a. Negative Control

The test substance vehicle was used as the concurrent negative control. The final concentration of vehicle in the treatment medium did not exceed 10% for vehicles such as water, buffer, or culture medium, and 1% for other solvents.

b. Positive Control

The positive controls were mitomycin-C (MMC) for the non-activated system and cyclophosphamide (CP) for the S9 activated system. No positive controls were used for numerical aberrations (polyploidy or endoreduplications, or both). Both positive controls were dissolved in sterile water. Two test concentrations of each positive control were used. The concentration range for MMC was 0.1–0.5 µg/mL, and for CP 1-30 µg/mL. Only one of the concentrations of each positive control were included in the cytogenetic analysis. The exposure periods for MMC were 4 and 20 hours, and the exposure period for CP was 4 hours. Exposure to the positive control substances were not included in the preliminary cytotoxicity portion of the study. The positive control did not contain any contaminants that would interfere with the conduct of the study, and was assumed to be stable under the conditions of administration. Concentration verification of the positive control substances was not conducted.

7. Identification of the Test System

Using computer generated labels or a permanent marker, each flask tube was labeled with the work request number, the Haskell number, dose level, replicate indicator (A or B), metabolic activation system (+/-S9), and exposure period.

8. Treatment of Target Cells

The day when the cells were first exposed to the test substance was designated as test day 0. Approximately 16-24 hours after seeding, the CHO cultures the culture medium were discarded and replaced with approximately 5 mL complete medium for the non-activated test condition, and 4 mL complete medium +1 mL of the S9 mixture for the activated test condition. The volumes were selected such that addition of the test substance volume (50 µL or 0.5 mL depending on the solvent) resulted in a total volume of approximately 5 mL.

Sets of duplicate cultures were then administered an aliquot of the test substance (at least five concentrations will be applied), the vehicle control, or at least one positive control substance concentrations (for the S9 activated and non-activated test condition). The cells were treated for 4- and 20-hours in the non-activated test condition, and for 4-hours in the S9 activated test condition. After completion of the 4-hour exposure periods only, the cells were collected by centrifugation, washed once with phosphate buffered saline, fed with complete medium and incubated until cell harvest. The incubations were conducted at 37 ± 2°C in a humidified atmosphere of 5 ± 1% CO_2 in air.

A concurrent cytotoxicity test determining total cell growth inhibition (%) relative to the solvent control was conducted for all assays and testing conditions.

9. Collection of Metaphase Cells and Cell Harvest

The cells were arrested in metaphase at approximately 18 hours after treatment initiation by adding Colcemid® to the cultures at a 0.1 µg/mL final concentration in the culture media. Approximately 20 hours after treatment initiation the cultures were collected by centrifugation and the medium was removed. The cells were treated with 0.075M KCl hypotonic buffer, fixed once in methanol and at least twice in methanol:glacial acetic acid (3:1 v/v), and stored (refrigerated or frozen) overnight or longer. To prepare slides, the cells were collected by centrifugation and resuspended in fresh fixative. At least two slides per culture were prepared by applying an aliquot of the fixed cells onto clean microscope slides and air drying them. The slides were stained by Giemsa and permanently mounted.

10. Identification of the Slides

Using computer generated labels or a permanent marker, each slide was labeled with the work request number, the Haskell number, dose level, replicate indicator (A or B), metabolic activation system (+/-S9), and exposure period.

11. Cytogenetic Analyses

Slides were scored coded. Metaphase cells were selected for scoring based on good chromosome morphology and staining characteristics. Only metaphase cells with 20 ± 2 centromeres were analyzed for structural aberrations. At least 200 metaphases per concentration level (100 from each duplicate culture), when available, were analyzed for structural aberrations.[5] Numerical aberrations were recorded as well. The number of metaphases evaluated per duplicate flask was less if the percentage of aberrant cells reached statistical significance when 25 cells were scored. Chromatid-type aberrations included chromatid and

isochromatid breaks and exchange figures. Chromosome-type aberrations included chromosome breaks and exchange figures. Pulverized chromosome(s) and cells, and severely damaged cells (i.e., cells with ≥10 aberrations per cell) were recorded, but not included in the analyses. The percentages of cells in metaphase per 2000 cells scored per concentration level (1000 from each duplicate culture) were determined. The XY coordinates for the microscope stage was recorded for cells with structural or numerical aberrations.

E. Criteria for Determination of a Valid Test

An assay was considered acceptable for evaluation of test results only if all of the following criteria were satisfied. The metabolically activated and non-activated assays of the test were independent and, if necessary, were repeated separately.

1. Vehicle Controls

The frequency of cells with structural chromosome aberrations must be less than approximately 5%.

2. Positive Controls

The percentage of cells with structural chromosome aberrations must be statistically significantly greater ($p \leq 0.05$, Fisher's exact test) than the vehicle control response.

F. Evaluation of Test Results and Statistical Analyses

The clastogenic potential of the test substance was assessed based on its ability to induce structural chromosome aberrations. The experimental unit is the cell; therefore the percentage of cells with structural aberrations was used for the assessment.

Data was evaluated using scientific judgment. Statistical analysis was used as a guide to determine whether or not the test substance induced a positive response. Interpretation of the statistical analysis also relied on additional considerations including the magnitude of the observed test substance response relative to the vehicle control response and the presence of a dose responsive trend. Statistical analysis consisted of a Cochran-Armitage test for dose responsiveness and Fisher's exact test to compare the percentage of cells with aberrations (or the percentage of cells with more than one aberration, if required) in the test substance treated groups with the vehicle control response.[6-7] At the discretion of the study director, statistical analyses were performed on the percentage of cells with numerical aberrations.

The following conditions were used as a guide to determine a positive response:

- A statistically significant increase ($p \leq 0.05$, Fisher's exact test) in the percentage of cells with structural aberrations was seen in one or more treatment groups relative to the vehicle control response.
- The observed increased frequencies was accompanied by a concentration-related increase.
- A statistically significant increase was observed at the highest dose only.

The following condition was used as a guide to determine an equivocal response:

- Results observed in any of the assays result in statistically significant elevations in structural chromosome aberrations at more than one test concentration level without demonstrating a dose-responsive trend.

The test substance was judged negative if the following condition is met:

- There was no statistically significant increase in the percentage of cells with structural aberrations in any treatment group relative to the vehicle control group.

G. Data Presentation

The data was summarized in tables containing, but not limited to, cell counts, cell growth inhibition, mitotic index, percent polyploidy/endoreduplication (numerical aberrations), number of cells analyzed, types of structural aberrations, frequencies of structural aberrations per cell, and the percentage of cells with structural aberrations. Chromatid and chromosome gaps were listed, but not added to the totals for structural aberration evaluation (gaps were not considered true structural damage).

RESULTS AND DISCUSSION

A. Solubility

DMSO was determined to be the solvent of choice based on the solubility of the test substance and compatibility with the target cells. The test substance formed a clear solution in the vehicle at the highest stock concentration prepared, 500 mg/mL

B. Preliminary Toxicity Assay

(Table 1)

Concentrations for the chromosome aberration assay were selected based on the results from a preliminary toxicity test assessing the cell growth inhibition relative to the vehicle control. The cultures were microscopically inspected for the extent of monolayer confluency relative to the solvent control. The data is on file, but not included in the report; the assessment is mainly an aid for study conduct. In the preliminary toxicity assay, the highest concentration tested was 810.5 µg/mL (10 mM), the guidelined limit dose for this test system (i.e., 5000 µg/mL or 10 mM, whichever is lower). CHO cells were exposed to nine concentrations of the test substance ranging from 162.1 to 810.5 µg/mL, as well as the vehicle control, in both the absence and presence of an exogenous metabolic activation system (Aroclor-induced S9) for 4 hours, or for 20 hours in the absence of S9 activation. The test substance was soluble in the vehicle and in the treatment medium at all concentration levels tested. Based on visual inspection, the pH of the highest test substance concentration in media was not significantly different than the pH of the vehicle control. Osmolality measurements were taken from the highest test substance concentration in media in connection with the chromosome aberration assay. Substantial toxicity (at least a 50% reduction in cell growth relative to the solvent control) was not observed at any

concentration level in any test system. Based on the findings from the preliminary toxicity assay, the highest concentration chosen for the chromosome aberration assay was 810.5 µg/mL for all three test systems.

C. Chromosome Aberration Assay

(Tables 2-8)

Based on the findings from the preliminary toxicity assay, the concentrations chosen for the chromosome aberration assay were 324.2, 486.3, 648.4, 729.5, and 810.5 µg/mL (10 mM) for all three test systems. The osmolality of the highest test substance concentration in media was not significantly different than the osmolality of the vehicle control. In the treatment medium of the highest concentration tested, 810.5 µg/mL, the osmolality was 451 and 478 mmol/kg in the non-activated and activated test system, respectively. The osmolality of the vehicle in the treatment medium was 475 and 481 mmol/kg in the non-activated and activated test system, respectively. No precipitation was observed in the treatment medium at any concentration level in any test system. The visual assessment of precipitation was done at the beginning and the end of the treatment periods. In addition, the uncoded slides were scanned by microscopic observation for the presence of mitotic cells, to ensure the selection of scorable test substance concentrations (data not shown).

The concurrent toxicity data are presented in Tables 2-4. The concentration levels 324.2, 648.4, and 810.5 µg/mL were selected for chromosome aberration analyses for the 4- and 20-hour non-activated test systems. For the 4-hour activated test system, test substance–related inhibition of mitotic activity was observed at all concentration levels (data not reported). A repeat test was conducted at concentration levels 1.86, 3.81, 7.62, 15.2, 30.4, 60.8, 121.6, 162.1, and 243.2 µg/mL Based on the concurrent toxicity assay (Table 3) and scanning the slides for mitotic activity (data not shown), the concentration levels 60.79, 121.6, and 162.1 µg/mL (2 mM)were selected for chromosome aberration analyses.

The individual treatment culture findings of the cytogenetic analysis of the non-activated 4-hour exposure group are presented in Table 5 and summarized by group in Table 8. At the highest test concentration evaluated microscopically for chromosome aberrations, 810.5 µg/mL, a 15% growth inhibition in relation to the vehicle was observed (Table 2). The mitotic index was not significantly different from the vehicle control. The percentage of cells with structural or numerical aberrations in the test substance-treated groups was not significantly increased above that of the vehicle control at any concentration ($p > 0.05$, Fisher's exact test). The Cochran-Armitage test for dose response was statistically significant ($p < 0.05$) for the percentage of cells with numerical aberrations. However, the observed percentage of cells with numerical aberrations are within the historical solvent control range of 0-5% for numerical aberrations. Therefore the results are not considered biologically significant. The percentage of cells with structurally damaged chromosomes in the MMC (positive control) treatment group (11%) was statistically significant.

The individual treatment culture findings of the cytogenetic analysis of the 4-hour S9 activated group are presented in Table 6 and summarized by group in Table 8. At the highest test concentration evaluated microscopically for chromosome aberrations, 162.1 µg/mL, a 49%

growth inhibition in relation to the vehicle was observed (Table 3). The mitotic index was not significantly different from the vehicle control. The percentage of cells with structural aberrations in the 121.6 and 162.1 µg/mL test substance-treated groups was statistically significant when compared to that of the solvent control ($p < 0.05$, Fisher's exact test). The Cochran-Armitage test for dose response was also statistically significant ($p < 0.05$). Although the observation of 4.5% of cells with structural aberrations at 121.6 µg/mL is within the historical solvent control range of 0-6% for structural aberrations, the 10.5% of cells with structural aberrations at 162.1 µg/mL, is above the historical solvent control range. Therefore the results are considered biologically significant. The percentage of cells with numerical chromosome aberrations in any test substance-treated groups was not significantly increased above that of the solvent control at any concentration ($p > 0.05$, Fisher's exact test. The observed increase in numerical aberrations at the 60.79 µg/mL concentration level is above the historical solvent control range of 0-4% for numerical aberrations, and the Cochran-Armitage test for dose response was not statistically significant ($p > 0.05$). Therefore the observed increase is considered biologically irrelevant. The percentage of cells with structurally damaged chromosomes in the CP (positive control) treatment group (9.5%) was statistically significant.

The individual treatment culture specific findings of the cytogenetic analysis of the non-activated 20-hour group are presented in Table 7 and summarized by group in Table 8. At the highest test concentration evaluated microscopically for chromosome aberrations, 810.5 µg/mL, a 23% growth inhibition in relation to the vehicle was observed (Table 4). The mitotic index was not significantly different from the vehicle control. The percentage of cells with structural or numerical aberrations in any test substance-treated groups was not significantly increased above that of the solvent control at any concentration ($p > 0.05$, Fisher's exact test). The Cochran-Armitage test for dose response was statistically significant ($p < 0.05$) for the percentage of cells with numerical aberrations. However, the observed percentages of cells with numerical aberrations are within the historical solvent control range of 0-5% for numerical aberrations. Therefore the results are not considered biologically significant. The percentage of cells with structurally damaged chromosomes in the MMC (positive control) treatment group (10%) was statistically significant.

CONCLUSION

All criteria for a valid study were met. Under the conditions of this study, H-26232 was found to induce structural chromosome aberrations in the *in vitro* mammalian chromosome aberration test in Chinese hamster ovary cells in the S9 activated test system, but not in the non-activated test system. H-26232 was found to be negative for the induction of numerical chromosome aberrations in both the non-activated and S9 activated test systems. The test substance was concluded as positive in this *in vitro* test.

RECORDS AND SAMPLE STORAGE

The retention of an archive sample of the test substance will be the responsibility of the sponsor.

Specimens (if applicable), raw data, and the final report will be retained at Haskell Laboratory, Newark, Delaware, or at Iron Mountain Records Management, Wilmington, Delaware, and will be returned to the sponsor within 6 months after the final report issues.

REFERENCES

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TABLES

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EXPLANATORY NOTES

ABBREVIATIONS:

% Aberrant Cells	numerical cells include polyploid and endoreduplicated cells; structural cells exclude cells with only gaps
Aberrations Per Cell	severely damaged cells were counted as 10 aberrations
Br	break
Cell Growth Index	cells per flask treated group/cells per flask control group), expressed as a percentage
Cell Growth Inhibition	100% - % cell growth index; not calculated for negative controls.
CHO	chinese hamster ovary
Chromatid Breaks	include chromatid and isochromatid breaks and fragments (Br); chromatid exchange figures (Ex) include quadriradials, triradials and complex rearrangements
Chromosome Breaks	include breaks and acentric fragments (Br); Dic, dicentric chromosome
Ex	exchange
Mitotic Index	(cells in mitosis / # cells scored) x 100
Percent Change	(treatment mitotic index - control mitotic index)/control mitotic index, expressed as a percentage
SD	standard deviation
Severely Damaged Cells	includes cells with one or more pulverized chromosome and cells with 10 or more structural aberrations

Table 1: Preliminary toxicity test using H-26232 in the absence or presence of exogenous metabolic activation

	4 Hours						20 Hours		
	S9-			S9+			S9-		
Treatment[a] (µg/mL)	Cell Count (10^6)	Cell Growth Index (%)	Cell Growth Inhibition (%)	Cell Count (10^6)	Cell Growth Index (%)	Cell Growth Inhibition (%)	Cell Count (10^6)	Cell Growth Index (%)	Cell Growth Inhibition (%)
Vehicle[b]	1.03	100	NA	0.88	100	NA	1.02	100	NA
162.1	1.04	101	-1	0.90	102	-2	0.96	94	6
243.2	0.96	93	7	0.84	95	5	0.96	94	6
324.2	0.77	75	25	0.86	98	2	0.93	91	9
405.2	0.81	79	21	0.78	89	11	0.92	90	10
486.3	0.82	80	20	0.86	98	2	0.88	86	14
567.4	0.92	89	11	0.84	95	5	0.85	83	17
648.4	0.90	87	13	0.78	89	11	0.89	87	13
729.5	0.85	83	17	0.79	90	10	0.85	83	17
810.5[c]	0.82	80	20	0.76	86	14	0.87	85	15

[a] CHO cells were treated at 37°C.
[b] DMSO
[c] Equivalent to a 10 mM concentration.

Table 2: Concurrent toxicity test using H-26232 in the absence of exogenous metabolic activation (4-hour treatment, 16-hour recovery period)

Treatment[a] (µg/mL)	Flask	Cell Count (10^6)	Cell Growth Index (%)	Cell Growth Inhibition (%)
Vehicle[b]	A	1.15	100	NA
	B	1.28		
324.2	A	1.18	98	2
	B	1.20		
486.3	A	1.12	91	9
	B	1.09		
648.4	A	1.16	95	5
	B	1.15		
729.5	A	0.95	80	20
	B	0.99		
810.5[c]	A	0.99	85	15
	B	1.07		
MMC 0.2	A	0.86	69	31
	B	0.82		
MMC 0.4	A	0.76	61	39
	B	0.72		

[a] CHO cells were treated at 37°C.
[b] DMSO
[c] Equivalent to a 10 mM concentration.

Table 3: Concurrent toxicity test using H-26232 in the presence of exogenous metabolic activation (4-hour treatment, 16-hour recovery period)

Treatment[a] (µg/mL)	Flask	Cell Count (10^6)	Cell Growth Index (%)	Cell Growth Inhibition (%)
Vehicle[b]	A	1.29	100	NA
	B	1.29		
15.24	A	1.27	98	2
	B	1.27		
30.39	A	1.23	96	4
	B	1.24		
60.79	A	1.20	94	6
	B	1.23		
121.6	A	0.96	74	26
	B	0.94		
162.1	A	0.66	51	49
	B	0.66		
243.2[c]	A	0.68	54	46
	B	0.71		
CP 7.5	A	0.80	63	37
	B	0.83		
CP 10	A	0.76	58	42
	B	0.74		

[a] CHO cells were treated at 37°C.
[b] DMSO
[c] Equivalent to a 3 mM concentration.

Table 4: Concurrent toxicity test using H-26232 in the absence of exogenous metabolic activation (20-hour continuous treatment)

Treatment[a] (µg/mL)	Flask	Cell Count (10^6)	Cell Growth Index (%)	Cell Growth Inhibition (%)
Vehicle[b]	A	1.38	100	NA
	B	1.31		
324.2	A	1.30	98	2
	B	1.33		
486.3	A	1.07	81	19
	B	1.10		
648.4	A	1.07	79	21
	B	1.05		
729.5	A	1.07	75	25
	B	0.96		
810.5[c]	A	1.05	77	23
	B	1.02		
MMC 0.2	A	0.90	67	33
	B	0.89		
MMC 0.4	A	0.79	58	42
	B	0.77		

[a] CHO cells were treated at 37°C.
[b] DMSO
[c] Equivalent to a 10 mM concentration.

Table 5: Cytogenetic analysis of CHO cells treated with H-26232 in the absence of exogenous metabolic activation (4-hour treatment, 16-hour recovery period)

Treatment[a] (µg/mL)	Slide	Mitotic Index (%)	Cells Scored		% Aberrant Cells[b]		Total Number of Structural Aberrations					Severely Damaged Cells	Average Aberrations Per Cell
								Chromatid		Chromosome			
			Numerical	Structural	Numerical	Structural	Gaps	Br	Ex	Br	Ex		
Vehicle[c]	A	7.5	100	100	2	0	0	0	0	0	0	0	0.000
	B	7.9	100	100	0	0	0	0	0	0	0	0	0.000
324.2	A	6.6	100	100	0	0	0	0	0	0	0	0	0.000
	B	7.3	100	100	2	0	0	0	0	0	0	0	0.000
648.4	A	6.4	100	100	3	1	0	1	0	0	0	0	0.010
	B	5.8	100	100	1	0	0	0	0	0	0	0	0.000
810.5[d]	A	5.9	100	100	6	0	0	0	0	0	0	0	0.000
	B	6.2	100	100	4	0	0	0	0	0	0	0	0.000
MMC 0.4	A	4.1	25	25	0	12	0	5	7	0	0	0	0.480
	B	4.0	25	25	1	10	0	5	5	0	0	0	0.400

[a] CHO cells were treated at 37°C.
[b] Excluding cells with only gaps.
[c] DMSO
[d] Equivalent to a 10 mM concentration.

Table 6: Cytogenetic analysis of CHO cells treated with H-26232 in the presence of exogenous metabolic activation (4-hour treatment, 16-hour recovery period)

Treatment[a] (µg/mL)	Slide	Mitotic Index (%)	Cells Scored		% Aberrant Cells[b]		Total Number of Structural Aberrations					Severely Damaged Cells	Average Aberrations Per Cell
								Chromatid		Chromosome			
			Numerical	Structural	Numerical	Structural	Gaps	Br	Ex	Br	Ex		
Vehicle[c]	A	6.9	100	100	6	0	0	0	0	0	0	0	0.000
	B	8.4	100	100	4	0	0	0	0	0	0	0	0.000
60.79	A	8.2	100	100	7	1	0	0	0	0	1	0	0.010
	B	7.1	100	100	6	0	0	0	0	0	0	0	0.000
121.6	A	6.6	100	100	6	5	0	3	1	0	2	0	0.060
	B	7.4	100	100	2	4	1	2	2	0	0	0	0.040
162.1[d]	A	7.4	100	100	5	9	0	6	3	0	0	0	0.090
	B	7.0	100	100	5	12	0	4	10	0	0	0	0.140
CP 7.5	A	5.2	25	25	0	9	0	5	6	0	0	0	0.440
	B	5.3	25	25	0	10	0	7	5	0	0	0	0.480

[a] CHO cells were treated at 37°C.
[b] Excluding cells with only gaps.
[c] DMSO
[d] Equivalent to a 2 mM concentration.

Table 7: Cytogenetic analysis of CHO cells treated with H-26232 in the absence of exogenous metabolic activation (20-hour continuous treatment)

Treatment[a] (µg/mL)	Slide	Mitotic Index (%)	Cells Scored		% Aberrant Cells[b]		Total Number of Structural Aberrations					Severely Damaged Cells	Average Aberrations Per Cell
								Chromatid		Chromosome			
			Numerical	Structural	Numerical	Structural	Gaps	Br	Ex	Br	Ex		
Vehicle[c]	A	7.2	100	100	1	0	0	0	0	0	0	0	0.000
	B	7.4	100	100	0	0	0	0	0	0	0	0	0.000
324.2	A	7.0	100	100	3	0	0	0	0	0	0	0	0.000
	B	7.2	100	100	2	1	0	0	0	0	1	0	0.010
648.4	A	7.3	100	100	2	0	0	0	0	0	0	0	0.000
	B	7.2	100	100	5	0	0	0	0	0	0	0	0.000
810.5[d]	A	5.8	100	100	5	1	0	1	0	0	0	0	0.010
	B	6.5	100	100	4	2	0	2	0	0	0	0	0.020
MMC 0.4	A	3.8	25	25	0	10	0	3	7	0	2	0	0.480
	B	3.4	25	25	0	10	0	6	7	0	0	0	0.520

[a] CHO cells were treated at 37°C.
[b] Excluding cells with only gaps.
[c] DMSO
[d] Equivalent to a 10 mM concentration.

Table 8: Summary

Treatment[a] µg/mL	S9 Activation	Treatment Time	Mitotic Index (%)	Cells Scored[b]		Aberrations Per Cell		Cells with Aberrations	
				Numerical	Structural	Mean	SD	Numerical (%)	Structural (%)
Vehicle[c]	-S9	4	7.7	200	200	0.000	0.000	1.0	0.0
324.2	-S9	4	7.0	200	200	0.000	0.000	1.0	0.0
648.4	-S9	4	6.1	200	200	0.005	0.007	2.0	0.5
810.5[d]	-S9	4	6.1	200	200	0.000	0.000	5.0[e]	0.0
MMC 0.4	-S9	4	4.1	50	50	0.440	0.057	0.5	11.0[f]
Vehicle	+S9	4	7.7	200	200	0.000	0.000	5.0	0.0
60.79	+S9	4	7.7	200	200	0.005	0.007	6.5	0.5
121.6	+S9	4	7.0	200	200	0.050	0.014	4.0	4.5[e,f]
162.1[g]	+S9	4	7.2	200	200	0.115	0.035	4.5	10.5[e,f]
CP 7.5	+S9	4	5.3	50	50	0.460	0.028	0.0	9.5[f]
Vehicle	-S9	20	7.3	200	200	0.000	0.000	0.5	0.0
324.2	-S9	20	7.1	200	200	0.005	0.007	2.5	0.5
648.4	-S9	20	7.3	200	200	0.000	0.000	3.5[e]	0.0
810.5	-S9	20	6.2	200	200	0.015	0.007	4.5[e]	1.5
MMC 0.4	-S9	20	3.6	50	50	0.500	0.028	0.0	10.0[f]

[a] CHO cells were treated at 37°C.
[b] Excluding cells with only gaps.
[c] DMSO
[d] Equivalent to a 10 mM concentration.
[e] Statistically significant difference from control at $p < 0.05$ by Cochran-Armitage test.
[f] Statistically significant difference from control at $p < 0.05$ by Fisher's test.
[g] Equivalent to a 2 mM concentration.

APPENDICES

Appendix A:

Historical Control Data

HISTORICAL CONTROL DATA[a]

Historical Values	Non-Activated Test System		S9 Activated Test System	
	Solvent Control	Positive Control[b]	Solvent Control	Positive Control[c]
Structural Chromosome Aberrations				
Mean	1.67	30.7	2.0	24.25
Standard Deviation	1.18	1.24	1.41	18.27
Range	0 – 5	10 – 51	0 – 6	7 – 68
Numerical Chromosome Aberrations				
Mean	2.17	0.17	2.33	0.08
Standard Deviation	1.53	0.59	1.65	0.28
Range	0 – 5	0 – 1	0 – 4	0 – 1

[a] Data are based on studies conducted since 2002. Data include all control solvents or diluents and metabolic activation systems based on Aroclor-induced rat liver S9.
[b] Mitomycin C (MMC)
[c] Cyclophosphamide (CP)